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(54) Use of trans-trans isomers of conjugated linoleic acid

(57) CLA -isomer mixtures rich in trans/trans isomers were found to have excellent anti-inflammatory properties and can be used for these purposes in foods or in food supplements, simultaneously these isomers improve the product performance of many food products.

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Description

[0001] Conjugated linoleic acid is indicated in many literature references as a composition having a number of health benefits. In particular WARF filed many patent applications on these benefits.

[0002] Conjugated linoleic acid however is not a single compound, but consists of a great number of isomers. These isomers include isomers wherein the 2 double bonds have different positions in the fatty acid molecule, but also isomers based on cis/trans isomerism.

[0003] So far all references on conjugated linoleic acid strongly suggest that the health effects that are reported are due to the presence of at least a cis double bond in the system. In particular the $cis^{9}trans^{11}$ and $trans^{10}cis^{12}$ isomers are held responsible for the beneficial effects. This can be concluded from eg. the following documents: WO 99/29317; WO 96/34855 but also from scientific publications such as, Poultry Science 72 (1993) p.1301-1305 or J Lipid Research 40 (1999) p.1426 - 33 or Lipids 33 (5) 1998, p.521-7. In contrast, Lipids 34 (3), 1999, p.235 -41, shows that $trans^{9}trans^{11}$ CLA has no biological effect at all.

[0004] We studied whether these effects really are due to the isomers mentioned above or whether other isomers from CLA either contribute to these known effects or display novel effects. This study resulted in the surprising finding that in in-vitro tests, in particular the tt-CLA compounds (ie conjugated linoleic acid isomers wherein all bonds are trans double bonds) have beneficial properties, in particular anti-inflammatory properties. Moreover we found that a tt CLA isomer mixture containing predominantly $trans^{9}trans^{11}$ and $trans^{10}trans^{12}$ isomers displayed the strongest biological effect.

[0005] Therefore our invention concerns in the first instance the use of a composition comprising 1 to 100, preferably 5 to 90, most preferably 20 to 85 wt% (based on total composition) of isomers of trans/trans conjugated linoleic acid (=tt-CLA) as measured by ^{13}C-magnetic resonance, wherein the tt-CLA is applied as an anti-inflammatory agent.

[0006] According to another embodiment of our invention these compositions can be applied as an additive in a food product or as a food supplement, preferably in encapsulated form in order to provide these products with anti-inflammatory properties. The use of these tt-rich CLA compositions simultaneously was found to result in an improvement of the physical properties, such as improved hardness, texture, firmness and overrun of food products.

[0007] The mouthfeel, oral meltdown and flavour release are not negatively affected by the tt-CLA. In addition, the products are easier to process and have better aeration properties.

[0008] It is preferred that the tt-CLA compositions according to the invention have a high relative tt-CLA content. Therefore we prefer to use a composition, that comprises the tt-CLA isomers as measured by ^{13}C magnetic resonance techniques in amounts of 5 to 90 wt% (on total composition), while the CLA isomers $cis^{9}trans^{11}$ and $trans^{10}cis^{12}$ are present in a weight ratio of:
$(t^{9}t^{11}+t^{10}t^{12})$: $(c^{9}t^{11} + t^{10}c^{12})$ of more than 3:1, preferably more than 5:1, most preferably more than 7.5:1.

[0009] The ^{13}C magnetic resonance technique for the determination of the isomer distribution in the CLA is known from Davis, A.L. et al Chemistry and physics of Lipids, 97, (1999) p.155-65.

[0010] The tt-rich CLA mixtures that can be applied according to the invention can be selected from free fatty acids, mono-, di- or triglycerides and alkylesters from CLA.

[0011] Although tt-CLA always will be produced in some (minor) amounts during the production of CLA isomer mixtures it would be very beneficial if concentrates would be available, as these would ease the dosing into foods and in particular would enable to make effective food supplements. Therefore we studied whether we could obtain concentrates of tt-CLA. This study resulted in another embodiment of our invention ie. in compositions with anti-inflammatory properties comprising CLA isomers, wherein the composition comprises more than 10 wt%, preferably more than 25 wt%, most preferably more than 50 wt% of CLA-isomers from which more than 55, preferably more than 60, most preferably more than 65 wt% (on total of CLA isomers) are tt-CLA isomers as measured by ^{13}C-magnetic resonance techniques.

[0012] Preferred compositions that we obtained are compositions, wherein the tt-CLA isomers $t^{9}t^{11}$ and $t^{10}t^{12}$ are present in a weight ratio $t^{9}t^{11}$:$t^{10}t^{12}$ of less than 2.3:1, preferably less than 1.8:1, most preferably less than 1.0:1 (as measured by ^{13}C magnetic resonance techniques).

[0013] Other preferred compositions comprise in addition to the tt-isomers $c^{9}t^{11}$-and $t^{10}c^{12}$-CLA isomers in a weight ratio of (total $t^{9}t^{11} + t^{10}t^{12}$): $(c^{9}t^{11} + t^{10}c^{12})$ of more than 3:1, preferably more than 5:1, most preferably more than 7.5:1 as measured by ^{13}C-magnetic resonance technology.

[0014] The tt-CLA composition according to the invention can suitably be applied in food products. Although the tt-CLA compositions could be present in any form (ie free acid / glycerides / alkylesters with alkyl groups with 2-20, preferably 2-8 carbon atoms) we prefer to use these compositions as glycerides because in that way the best oral mouthfeel properties of the food can be achieved.

[0015] Part of our invention therefore also are food products, preferably selected from the group consisting of confectionery products, spreads, sauces, dressings, mayonnaise, cheese, ice cream, doughs, baked bakery products, fillings and creams containing an effective amount of a composition comprising 1 to 100 wt% of tt-CLA, preferably in

the form of a mono- and/or di- and/or triglyceride. Most preferred are food products containing an effective amount of the preferred CLA-composition as defined above. An effective amount is defined as that amount, that corresponds with the recommended daily amount as achievable in 1-5 foodservices per day.

[0016] A very convenient method to administer an effective dose of our tt-CLA to the users is by providing our users with food supplements containing an effective dose of the tt-CLA. Therefore part of our invention is also food supplements, wherein the tt-rich CLA is encapsulated in a food grade encapsulating material, such as sugar, lactose, starch, modified starch, gelatine, cyclodextrin, proteins and cellulose.

[0017] The food supplements can also be fortified with other food ingredients. These ingredients can be selected from the group consisting of vitamins A, B, C, D, E, K, minerals such as calcium, potassium, magnesium, iron, copper, zinc, selenium and anti-oxidants such as tocopherols, polyphenols.

[0018] The tt-rich CLA isomer composition can be made by a process

i) wherein a composition with a total unconjugated C18:2 content of more than 55 wt% is conjugated and isomerized subjecting it to a catalytic treatment with a Sulphur-Nickel catalyst at a temperature of 100 to 180 °C in the absence of hydrogen

ii) the product formed in step i) is subjected to a solvent fractionation using acetone in a weight ratio of acetone to CLA product of 10:1 to 1:1 at a temperature of -25 to -100 °C, whereupon a stearine (or top) fraction is isolated as product according to the invention.

[0019] An alternative process for making these compositions comprises an enrichment of a mixture comprising different isomers of conjugated linoleic acid in the tt-CLA isomers wherein a mixture comprising at least 1 wt% of tt-CLA in admixture with other CLA isomers is subjected to an enzymic conversion, selected from the group consisting of i) partial hydrolysis of a glyceride mixture or of a mixture of esters of short chain alcohols, ii) partial esterification of a mixture of free fatty acids with glycerol or with a glyceride, iii) partial glycerolysis of a glyceride mixture and iv) partial esterification of an alcohol with free CLA isomers, while using an enzyme that can discriminate tt-CLA from other CLA isomers, whereupon the reaction product is separated by physical means separating a fraction that is enriched in tt-CLA as reaction product.

[0020] Enzymes that can be applied in above process can be selected from the group consisting of Candida rugosa lipase; Lipase QL; Lipase SL, Lipase OF and Geotrichum candidum B lipase, Lypozyme IM, Lipozyme M.

[0021] According to another alternative process, as illustrated in example 4, tt-CLA can be made effectively by subjecting a mixture of CLA isomers (obtained by base treatment of an oil, rich in linoleic acid in propylene glycol) to microwaves, It was found that microwaving during 3-10 min using 700-900 watts already resulted in high conversion rates to tt-CLA.

EXPERIMENTAL PART

[0022] The anti-inflammatory effects were determined by *in vitro* tests wherein the production Prostaglandin E2 (=PGE2) by the human skin fibroblasts, blood vessel endothelial cells (HUVECS=Human Umbilicial Vein Endothelial Cells) and blood is measured following stimulation by the inflammatory modulus PMA. A reduction of the levels of PGE2 is indicative for the anti-inflammatory effect.

[0023] Primary human foreskin fibroblasts at passage 2 (P2) were seeded into 96-well plates at 10000 cells/well and maintained for 24 hours in an atmosphere of 5% carbon dioxide in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum. Enriched tt-CLA (containing 84 wt% of tt-CLA and having a ratio ($t^9t^{11}+t^{10}t^{12}$) : ($c^9t^{11}+t^{10}c^{12}$) of 15 was added to fresh cell media in ethanol (final concentration 1%) in triplicate and incubated for a further 24 hours. Phorbal myristate acetate (PMA, Sigma) in ethanol/cell media was added to the media (final concentration 10nm) and the cells incubated for a futher 24 hours. PMA represents an external stressor which induces oxidative stress and inflammatory responses in cells. The media was then analysed immediately as described below.

[0024] **Prostaglandin E2 (PGE2) assay** Volumes of 50 µl culture medium were taken for PGE2 assay after gently shaking the culture plate. PGE2 levels in the medium were determined with a Biotrak PGE2 immunoassay kit (Amersham, UK). The assay is based on the competition between unlabelled PGE2 in the sample and a fixed quantity of horseradish peroxidase labelled PGE2 for a limited amount of fixed PGE2 specific antibody. Concentrations of unlabelled sample PGE2 were determined according to a standard curve which was obtained at the same time.

Results:

[0025] The below graph (Fig 1) demonstrates that challenging cells with an inflammatory stimulus such as PMA (Phorbol myristyl acetate) causes an increase in the inflammatory response as measured by prostaglandin E2 (PGE2) production. ttCLA, even at the levels of lOng/ml, dramatically reduces the inflammatory response as measured by

PGE2 production (Fig. 1).
- good anti-inflammatory activity.

Example 1

[0026] 10g of sunflower oil were mixed with lg of sulphur poisoned nickel hydrogenation catalyst. The mixture was stirred under a blanket of nitrogen at 180 °C in a glass vessel equipped with a magnetic stirrer.

[0027] After 6 hours a sample was removed extracted with a suitable solvent and filtered. The fatty acid composition of the triglyceride, as determined by FAME GC, contained 45% conjugated linoleic acid (CLA) of which 50% was tt CLA. The major tt isomers, determined by ^{13}C NMR, were trans^{9}trans^{11} and trans^{10}trans^{12} which together represented 60% of the total tt CLA.

Example 2

[0028] Conjugated linoleic acid was produced as described in example 1. An enriched product was made according to the following procedure. 5g of CLA was added to 30g of acetone in a stirred glass vessel and the temperature slowly reduced to -58 °C. The stearine fraction was isolated by vacuum filtration and washed with pre-cooled acetone. The fatty acid composition, determined by 13C-NMR, contained 33% CLA of which 86.6% was tt CLA. The major tt isomers, determined by ^{13}C NMR, were trans^{9}trans^{11} and trans^{10}trans^{12} which together represented 67% of the total tt CLA. This product was tested in above test on anti-inflammatory properties.

Example 3

[0029] Conjugated linoleic acid was produced by alkali isomerisation as described previously, W09718320, to give a product that contained 80% CLA. The two main isomers, determined by FAME GC, were cis^{9}trans^{11} and trans^{10}cis^{12} which together represented 95% of the total CLA.

[0030] 20g of this material was heated to 250 °C for 4 hours under a blanket of nitrogen. The fatty acid composition, determined by FAME GC, contained 67% CLA of which 30% was tt.

Example 4

[0031] 10.8 g of propylene glycol were stirred with 3.97 g of a 50 % aqueous KOH solution. The mixture was warmed to a temperature of 85 oC. 5.4 g of sunflower oil were added to this mixture and the mixture was stirred for 30 min. The mixture so obtained was placed in a microwave oven (Panasonic type NN5252B, 2450 MHz) and was subjected to microwaving at 850 Watts for 5.5 min. The mixture was cooled to 80 oC and 50 ml of diluted sulphuric acid (1:10) were added. The pH of the water layer was less then 3. The upperlayer was removed and washed with distilled water until neutral. The oil obtained was dried under vacuum. The FAME of the oil so obtained was:

C14:0	0.15 %
C16:0	3.95
C18:0	1.55
C18:1t	0.42
C18:1c	21.43
C18:2tt,ct	2.97
CLA 9c1t	6.59
CLA 11,13	4.32
CLA 10t12c	5.49
CLA tt	36.50
Balance other fatty acids.	

[0032] Thus the conjugation of the linoleic acid was about complete whereas from the CLA formed about 59 % was tt-CLA.

Example 5

[0033] Conjugated linoleic acid was produced as described in example 1. An enriched product was made by adding

2 g of this CLA to 2 g of water and 3,000 LU of lipase B from Geotrichum candidum. The mixture was stirred and maintained at 35 oC. The fatty acid composition of the glycerides as determined by FAME GC contained 50 wt% of CLA of which 57 % was tt CLA.

Definitions for products used in examples 6 and 7

[0034]

InES: Interesterified palm oil stearin/palmkernel stearin fat
Pof IV65 : Palm olein fraction with Iodine Value 65
SF : Sunflower oil
tt CLA : tt CLA containing oil
CN : Coconut oil

Example 6 Preparation of margarine

a) Formulation

[0035]

Aqueous Phase	
Water	18.48%
Potassium Sorbate	0.15
Citric Acid	0.07
SMP	1.0

Fat Phase	
Fat Blend	80.0
Hymono 8903	0.3

Fat Phase: - Product 1. 12% InEs, 88% SF (Control)
Product 2. 12% InEs, 10% tt CLA, 78% SF

b) Process Conditions

[0036] The process line was configured as: -
Premix - Pump - A_1-unit - C_1-unit - A_2-unit
Premix temperature was set at 60°C and 60-rpm stirrer speed. All units were set to 15°C, with shaft speeds set to 1000 rpm. Throughput was 50 g/min. using the constant displacement pump.
[0037] For all products a coarse premix was prepared by slowly adding the prepared aqueous phase to the oil phase in the premix tank. A 2 kg-batch was employed.
The mix was stirred for 15 minutes before pumping. After pumping the line was allowed to run for 15 minutes before any collection of product.
[0038] The following process parameters were noted: -

Product	A_1 exit (°C)	C_1 exit (°C)	A_2 exit (°C)	Line Pressure (bar)
Control	20.2	19.4	17.6	1.0
10% tt CLA	21.4	20.2	18.0	1.4

[0039] All tubs were placed at 5°C. After one day, one tub of each was transferred to each of 5°, 10°, 15° and 20°C for evaluations after one week.
[0040] All samples spread easily with no apparent water loss. All products are of excellent quality and displayed very good values for hardness (C-value), collar and conductivity at all storage temperatures (5, 10, 15 and 20°C).

5°C Storage

[0041]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	630	I/II	$<10^{-5}$
10% tt CLA	610	I/II	$<10^{-5}$

10° Storage

[0042]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	410	I	$<10^{-5}$
10% tt CLA	370	I	$<10^{-5}$

15°C Storage

[0043]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	340	I	$<10^{-5}$
10% tt CLA	390	I	$<10^{-5}$

20°C Storage

[0044]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	300	I	$<10^{-5}$
10% tt CLA	280	I	$<10^{-5}$

Example 7 Preparation of ice cream

[0045]

Table 1:

Recipe	
The following recipe was applied (cf table 1)	
Component	Wt%
Fat blend	10.0
Skimmed milk powder	10.0
Crystal sugar	12.0
Clear syrup	4.0
Dextrose anhydrate	2
Dimodan PVP	0.6
Water	61.4

[0046] The fat blends that were used are disclosed in table 2

Table 2:

Fat blend		
	Component	Wt%
Reference	POf IV65/ CN/ SF	30/20/50
Sample	POf IV65/CN/CLA tt containing oil	30/20/50

[0047] The sugar, milk powder and dextrose were mixed and added to the water. The mixture was heated to 70°C and the clear syrup was added. Next the fat blend and the emulsifier were added. The emulsion was stirred with an ultra-turrax, cooled down to 20°C and stirred again with the ultra-turrax. The emulsion stayed overnight in the refrigerator at 7°C. The batch ice cream machine was held for 24 hours at -28°C. The emulsion was stirred in the machine for 40 minutes until the temperature was at its lowest. The resulting ice cream was stored at -18°C for at least 3 days and was then evaluated.

[0048] From table 3 it can be concluded that the products according to the invention displayed a better hardness and a higher overrun.

Table 3:

	Reference	Product with CLA t,t
Overrun in %	6.7	14.4
hardness	52	173

Taste panel

[0049] Seven panellist tasted the firmness of sample in comparison to the reference. For 5 out of seven the sample with CLA t,t had a higher firmness.

1. Reference with SF - CLA tt			
	less/slower	equal	more/faster
Firmness		2	5

Claims

1. Use of a composition comprising 1 to 100, preferably 5 to 90, most preferably 20 to 85 wt% (on total composition) of isomers of trans/trans conjugated linoleic acid (=tt-CLA) as measured by ^{13}C-magnetic resonance, wherein the tt-CLA is applied as anti-inflammatory agent.

2. Use of a composition according to claim 1 wherein the composition, comprising the tt-CLA is applied as an additive in a food product or as food supplement, preferably in encapsulated form.

3. Use of a composition according to claims 1 and 2 wherein the composition, comprising the tt-CLA isomers contains as measured by ^{13}C magnetic resonance techniques 5 to 90 wt% (on total composition) of tt-CLA, while also the CLA isomers $cis^{9}trans^{11}$ and $trans^{10}cis^{12}$ are present in a weight ratio of (total $t^{9}t^{11}+t^{10}t^{12}$) to ($c^{9}t^{11}+t^{10}c^{12}$) of more than 3:1, preferably more than 5:1, most preferably more than 7.5:1.

4. Use of a composition according to claims 1 to 3 wherein the tt-CLA isomers are present either as free fatty acids, or as mono-, di-, and/or triglycerides or as alkylesters.

5. Composition with anti-inflammatory properties comprising CLA isomers, wherein the composition comprises more than 10 wt%, preferably more than 25 wt%, most preferably more than 50 wt% of CLA-isomers, from which more than 55, preferably more than 60, most preferably more than 65 wt% (on total CLA isomers) are tt-CLA isomers as measured by ^{13}C-magnetic resonance techniques.

6. Composition with anti inflammatory properties according to claim 5, wherein the composition comprises the tt-CLA isomers t^9t^{11} and $t^{10}t^{12}$ in a weight ratio t^9t^{11} to $t^{10}t^{12}$ of less than 2.3:1, preferably less than 1.8:1, most preferably less than 1.0:1 (as measures by ^{13}C magnetic resonance techniques).

7. Composition according to claims 5 or 6 wherein the composition also contains c^9t^{11} and $t^{10}c^{12}$-CLA isomers in a weight ratio of (total $t^9t^{11}+t^{10}t^{12}$) to ($c^9t^{11}+t^{10}c^{12}$) of more than 3:1, preferable more than 5:1, most preferable more than 7.5:1 as measured by ^{13}C-magnetic resonance technology.

8. Food products, preferably selected from the group consisting of confectionery products, spreads, sauces, dressings, mayonnaise, cheese, ice cream, doughs, baked bakery products, fillings, creams containing an effective amount of a composition comprising 1 to 100 wt% of tt-CLA, preferably in the form of a mono- and/or di- and/or triglyceride and most preferably containing an effective amount of the composition according to claims 5 to 7.

9. Food supplements containing an effective amount of a composition comprising 1 to 100 wt% of tt-CLA, preferably containing an effective amount of a composition according to claims 5 to 7.

10. Food supplements according to claim 9 wherein the tt-CLA rich composition is encapsulated in a food grade encapsulating material.

11. Food supplement according to claims 9 to 10 wherein the composition comprising the tt-CLA is fortified with other food ingredients selected from the group consisting of vitamins A, B, C, D, E, K, minerals such as calcium, potassium, magnesium, iron, copper, zinc, selenium and anti-oxidants such as tocopherols, polyphenols.

12. Process for the preparation of a composition according to claims 5 to 7, wherein:

    i) a composition with a total unconjugated C18:2 content of more than 55 wt% is conjugated and isomerized by subjecting it to a catalytic treatment with a Sulphur-Nickel catalyst at a temperature of 100 to 180 oC In the absence of hydrogen
    ii) the product formed in step i) is subjected to a solvent fractionation using acetone in a weight ratio of acetone to CLA product of 10:1 to 1:1 at a temperature of -25 to -100 °C, whereupon a stearine (or top) fraction is isolated as product according to claims 5 to 6.

13. Process for the enrichment of a mixture comprising different isomers of conjugated linoleic acid in the tt-CLA isomers wherein a mixture comprising at least 1 wt% of tt-CLA in admixture with other CLA isomers is subjected to an enzymic conversion, selected from the group consisting of i) partial hydrolysis of a glyceride mixture or of a mixture of esters of short chain alcohols, ii) partial esterification of a mixture of free fatty acids with glycerol or with a glyceride, iii) partial glycerolysis of a glyceride mixture and iv) partial esterification of an alcohol with free CLA isomers, while using an enzyme that can discriminate tt-CLA from other CLA isomers, whereupon the reaction product is separated by physical means separating a fraction that is enriched in tt-CLA as reaction product.

14. Process according to claim 13 wherein the enzyme is selected from the group consisting of: Candida rugosa lipase, Lipase QL, Lipase SL, Lipase OF, Geotrichum candidum B lipase, Lypozyme IM and lypozyme M.

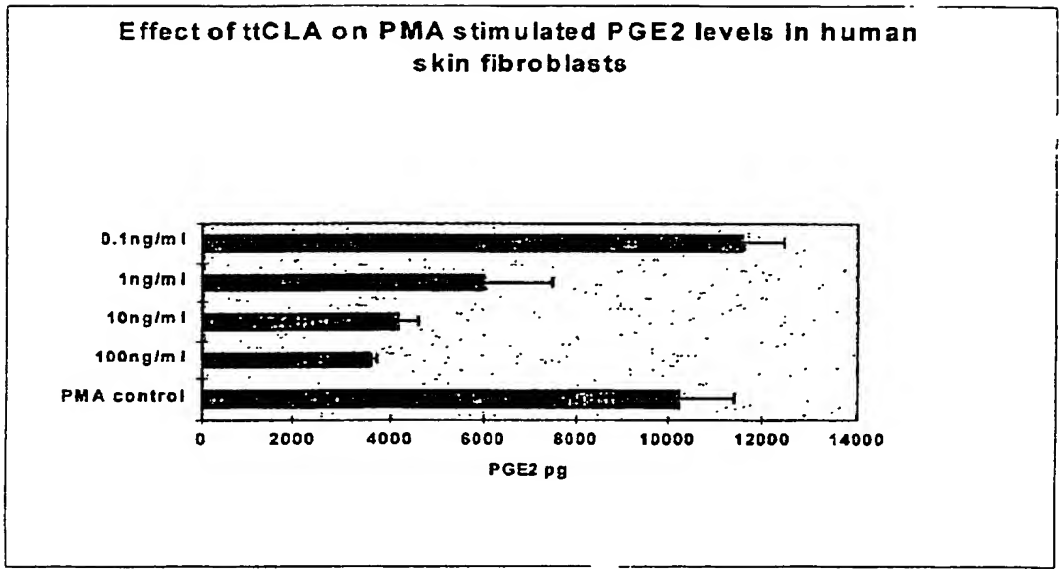

Fig. 1 Effect of ttCLA on PGE2 levels.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 20 3510

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	2 March 2001	Bendl, E

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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EUROPEAN SEARCH REPORT

Application Number
EP 00 20 3510

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(54) Use of trans-trans isomers of conjugated linoleic acid

(57) CLA -isomer mixtures rich in trans/trans isomers were found to have excellent anti-inflammatory properties and can be used for these purposes in foods or in food supplements, simultaneously these isomers improve the product performance of many food products.

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Description

[0001] Conjugated linoleic acid is indicated in many literature references as a composition having a number of health benefits. In particular WARF filed many patent applications on these benefits.

[0002] Conjugated linoleic acid however is not a single compound, but consists of a great number of isomers. These isomers include isomers wherein the 2 double bonds have different positions in the fatty acid molecule, but also isomers based on cis/trans isomerism.

[0003] So far all references on conjugated linoleic acid strongly suggest that the health effects that are reported are due to the presence of at least a cis double bond in the system. In particular the $cis^{9}trans^{11}$ and $trans^{10}cis^{12}$ isomers are held responsible for the beneficial effects. This can be concluded from eg. the following documents: WO 99/29317; WO 96/34855 but also from scientific publications such as, Poultry Science 72 (1993) p.1301-1305 or J Lipid Research 40 (1999) p.1426 - 33 or Lipids 33 (5) 1998, p.521-7. In contrast, Lipids 34 (3), 1999, p.235 -41, shows that $trans^{9}trans^{11}$ CLA has no biological effect at all.

[0004] We studied whether these effects really are due to the isomers mentioned above or whether other isomers from CLA either contribute to these known effects or display novel effects. This study resulted in the surprising finding that in in-vitro tests, in particular the tt-CLA compounds (ie conjugated linoleic acid isomers wherein all bonds are trans double bonds) have beneficial properties, in particular anti-inflammatory properties. Moreover we found that a tt CLA isomer mixture containing predominantly $trans^{9}trans^{11}$ and $trans^{10}trans^{12}$ isomers displayed the strongest biological effect.

[0005] Therefore our invention concerns in the first instance the use of a composition comprising 1 to 100, preferably 5 to 90, most preferably 20 to 85 wt% (based on total composition) of isomers of trans/trans conjugated linoleic acid (=tt-CLA) as measured by ^{13}C-magnetic resonance, wherein the tt-CLA is applied as an anti-inflammatory agent.

[0006] According to another embodiment of our invention these compositions can be applied as an additive in a food product or as a food supplement, preferably in encapsulated form in order to provide these products with anti-inflammatory properties. The use of these tt-rich CLA compositions simultaneously was found to result in an improvement of the physical properties, such as improved hardness, texture, firmness and overrun of food products.

[0007] The mouthfeel, oral meltdown and flavour release are not negatively affected by the tt-CLA. In addition, the products are easier to process and have better aeration properties.

[0008] It is preferred that the tt-CLA compositions according to the invention have a high relative tt-CLA content. Therefore we prefer to use a composition, that comprises the tt-CLA isomers as measured by ^{13}C magnetic resonance techniques in amounts of 5 to 90 wt% (on total composition), while the CLA isomers $cis^{9}trans^{11}$ and $trans^{10}cis^{12}$ are present in a weight ratio of:
$(t^{9}t^{11}+t^{10}t^{12})$: $(c^{9}t^{11} + t^{10}c^{12})$ of more than 3:1, preferably more than 5:1, most preferably more than 7.5:1.

[0009] The ^{13}C magnetic resonance technique for the determination of the isomer distribution in the CLA is known from Davis, A.L. et al Chemistry and physics of Lipids, 97, (1999) p.155-65.

[0010] The tt-rich CLA mixtures that can be applied according to the invention can be selected from free fatty acids, mono-, di- or triglycerides and alkylesters from CLA.

[0011] Although tt-CLA always will be produced in some (minor) amounts during the production of CLA isomer mixtures it would be very beneficial if concentrates would be available, as these would ease the dosing into foods and in particular would enable to make effective food supplements. Therefore we studied whether we could obtain concentrates of tt-CLA. This study resulted in another embodiment of our invention ie. in compositions with anti-inflammatory properties comprising CLA isomers, wherein the composition comprises more than 10 wt%, preferably more than 25 wt%, most preferably more than 50 wt% of CLA-isomers from which more than 55, preferably more than 60, most preferably more than 65 wt% (on total of CLA isomers) are tt-CLA isomers as measured by ^{13}C-magnetic resonance techniques.

[0012] Preferred compositions that we obtained are compositions, wherein the tt-CLA isomers $t^{9}t^{11}$ and $t^{10}t^{12}$ are present in a weight ratio $t^{9}t^{11}$:$t^{10}t^{12}$ of less than 2.3:1, preferably less than 1.8:1, most preferably less than 1.0:1 (as measured by ^{13}C magnetic resonance techniques).

[0013] Other preferred compositions comprise in addition to the tt-isomers $c^{9}t^{11}$-and $t^{10}c^{12}$-CLA isomers in a weight ratio of (total $t^{9}t^{11} + t^{10}t^{12}$): $(c^{9}t^{11} + t^{10}c^{12})$ of more than 3:1, preferably more than 5:1, most preferably more than 7.5:1 as measured by ^{13}C-magnetic resonance technology.

[0014] The tt-CLA composition according to the invention can suitably be applied in food products. Although the tt-CLA compositions could be present in any form (ie free acid / glycerides / alkylesters with alkyl groups with 2-20, preferably 2-8 carbon atoms) we prefer to use these compositions as glycerides because in that way the best oral mouthfeel properties of the food can be achieved.

[0015] Part of our invention therefore also are food products, preferably selected from the group consisting of confectionery products, spreads, sauces, dressings, mayonnaise, cheese, ice cream, doughs, baked bakery products, fillings and creams containing an effective amount of a composition comprising 1 to 100 wt% of tt-CLA, preferably in

the form of a mono- and/or di- and/or triglyceride. Most preferred are food products containing an effective amount of the preferred CLA-composition as defined above. An effective amount is defined as that amount, that corresponds with the recommended daily amount as achievable in 1-5 foodservices per day.

[0016] A very convenient method to administer an effective dose of our tt-CLA to the users is by providing our users with food supplements containing an effective dose of the tt-CLA. Therefore part of our invention is also food supplements, wherein the tt-rich CLA is encapsulated in a food grade encapsulating material, such as sugar, lactose, starch, modified starch, gelatine, cyclodextrin, proteins and cellulose.

[0017] The food supplements can also be fortified with other food ingredients. These ingredients can be selected from the group consisting of vitamins A, B, C, D, E, K, minerals such as calcium, potassium, magnesium, iron, copper, zinc, selenium and anti-oxidants such as tocopherols, polyphenols.

[0018] The tt-rich CLA isomer composition can be made by a process

i) wherein a composition with a total unconjugated C18:2 content of more than 55 wt% is conjugated and isomerized subjecting it to a catalytic treatment with a Sulphur-Nickel catalyst at a temperature of 100 to 180 °C in the absence of hydrogen

ii) the product formed in step i) is subjected to a solvent fractionation using acetone in a weight ratio of acetone to CLA product of 10:1 to 1:1 at a temperature of -25 to -100 °C, whereupon a stearine (or top) fraction is isolated as product according to the invention.

[0019] An alternative process for making these compositions comprises an enrichment of a mixture comprising different isomers of conjugated linoleic acid in the tt-CLA isomers wherein a mixture comprising at least 1 wt% of tt-CLA in admixture with other CLA isomers is subjected to an enzymic conversion, selected from the group consisting of i) partial hydrolysis of a glyceride mixture or of a mixture of esters of short chain alcohols, ii) partial esterification of a mixture of free fatty acids with glycerol or with a glyceride, iii) partial glycerolysis of a glyceride mixture and iv) partial esterification of an alcohol with free CLA isomers, while using an enzyme that can discriminate tt-CLA from other CLA isomers, whereupon the reaction product is separated by physical means separating a fraction that is enriched in tt-CLA as reaction product.

[0020] Enzymes that can be applied in above process can be selected from the group consisting of Candida rugosa lipase; Lipase QL; Lipase SL, Lipase OF and Geotrichum candidum B lipase, Lypozyme IM, Lipozyme M.

[0021] According to another alternative process, as illustrated in example 4, tt-CLA can be made effectively by subjecting a mixture of CLA isomers (obtained by base treatment of an oil, rich in linoleic acid in propylene glycol) to microwaves, It was found that microwaving during 3-10 min using 700-900 watts already resulted in high conversion rates to tt-CLA.

EXPERIMENTAL PART

[0022] The anti-inflammatory effects were determined by *in vitro* tests wherein the production Prostaglandin E2 (=PGE2) by the human skin fibroblasts, blood vessel endothelial cells (HUVECS=Human Umbilicial Vein Endothelial Cells) and blood is measured following stimulation by the inflammatory modulus PMA. A reduction of the levels of PGE2 is indicative for the anti-inflammatory effect.

[0023] Primary human foreskin fibroblasts at passage 2 (P2) were seeded into 96-well plates at 10000 cells/well and maintained for 24 hours in an atmosphere of 5% carbon dioxide in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum. Enriched tt-CLA (containing 84 wt% of tt-CLA and having a ratio ($t^9t^{11}+t^{10}t^{12}$) : ($c^9t^{11}+t^{10}c^{12}$) of 15 was added to fresh cell media in ethanol (final concentration 1%) in triplicate and incubated for a further 24 hours. Phorbal myristate acetate (PMA, Sigma) in ethanol/cell media was added to the media (final concentration 10nm) and the cells incubated for a futher 24 hours. PMA represents an external stressor which induces oxidative stress and inflammatory responses in cells. The media was then analysed immediately as described below.

[0024] **Prostaglandin E2 (PGE2) assay** Volumes of 50 µl culture medium were taken for PGE2 assay after gently shaking the culture plate. PGE2 levels in the medium were determined with a Biotrak PGE2 immunoassay kit (Amersham, UK). The assay is based on the competition between unlabelled PGE2 in the sample and a fixed quantity of horseradish peroxidase labelled PGE2 for a limited amount of fixed PGE2 specific antibody. Concentrations of unlabelled sample PGE2 were determined according to a standard curve which was obtained at the same time.

Results:

[0025] The below graph (Fig 1) demonstrates that challenging cells with an inflammatory stimulus such as PMA (Phorbol myristyl acetate) causes an increase in the inflammatory response as measured by prostaglandin E2 (PGE2) production. ttCLA, even at the levels of lOng/ml, dramatically reduces the inflammatory response as measured by

PGE2 production (Fig. 1) .
- good anti-inflammatory activity.

Example 1

[0026] 10g of sunflower oil were mixed with lg of sulphur poisoned nickel hydrogenation catalyst. The mixture was stirred under a blanket of nitrogen at 180 °C in a glass vessel equipped with a magnetic stirrer.

[0027] After 6 hours a sample was removed extracted with a suitable solvent and filtered. The fatty acid composition of the triglyceride, as determined by FAME GC, contained 45% conjugated linoleic acid (CLA) of which 50% was tt CLA. The major tt isomers, determined by ^{13}C NMR, were trans^{9}trans^{11} and trans^{10}trans^{12} which together represented 60% of the total tt CLA.

Example 2

[0028] Conjugated linoleic acid was produced as described in example 1. An enriched product was made according to the following procedure. 5g of CLA was added to 30g of acetone in a stirred glass vessel and the temperature slowly reduced to -58 °C. The stearine fraction was isolated by vacuum filtration and washed with pre-cooled acetone. The fatty acid composition, determined by 13C-NMR, contained 33% CLA of which 86.6% was tt CLA. The major tt isomers, determined by ^{13}C NMR, were trans^{9}trans^{11} and trans^{10}trans^{12} which together represented 67% of the total tt CLA. This product was tested in above test on anti-inflammatory properties.

Example 3

[0029] Conjugated linoleic acid was produced by alkali isomerisation as described previously, W09718320, to give a product that contained 80% CLA. The two main isomers, determined by FAME GC, were cis^{9}trans^{11} and trans^{10}cis^{12} which together represented 95% of the total CLA.

[0030] 20g of this material was heated to 250 °C for 4 hours under a blanket of nitrogen. The fatty acid composition, determined by FAME GC, contained 67% CLA of which 30% was tt.

Example 4

[0031] 10.8 g of propylene glycol were stirred with 3.97 g of a 50 % aqueous KOH solution. The mixture was warmed to a temperature of 85 oC. 5.4 g of sunflower oil were added to this mixture and the mixture was stirred for 30 min. The mixture so obtained was placed in a microwave oven (Panasonic type NN5252B, 2450 MHz) and was subjected to microwaving at 850 Watts for 5.5 min. The mixture was cooled to 80 oC and 50 ml of diluted sulphuric acid (1:10) were added. The pH of the water layer was less then 3. The upperlayer was removed and washed with distilled water until neutral. The oil obtained was dried under vacuum. The FAME of the oil so obtained was:

C14:0	0.15 %
C16:0	3.95
C18:0	1.55
C18:lt	0.42
C18:1c	21.43
C18:2tt,ct	2.97
CLA 9cllt	6.59
CLA 11,13	4.32
CLA 10tl2c	5.49
CLA tt	36.50
Balance other fatty acids.	

[0032] Thus the conjugation of the linoleic acid was about complete whereas from the CLA formed about 59 % was tt-CLA.

Example 5

[0033] Conjugated linoleic acid was produced as described in example 1. An enriched product was made by adding

2 g of this CLA to 2 g of water and 3,000 LU of lipase B from Geotrichum candidum. The mixture was stirred and maintained at 35 oC. The fatty acid composition of the glycerides as determined by FAME GC contained 50 wt% of CLA of which 57 % was tt CLA.

Definitions for products used in examples 6 and 7

[0034]

InES: Interesterified palm oil stearin/palmkernel stearin fat
Pof IV65 : Palm olein fraction with Iodine Value 65
SF : Sunflower oil
tt CLA : tt CLA containing oil
CN : Coconut oil

Example 6 Preparation of margarine

a) Formulation

[0035]

Aqueous Phase	
Water	18.48%
Potassium Sorbate	0.15
Citric Acid	0.07
SMP	1.0

Fat Phase	
Fat Blend	80.0
Hymono 8903	0.3

Fat Phase: - Product 1. 12% InEs, 88% SF (Control)
Product 2. 12% InEs, 10% tt CLA, 78% SF

b) Process Conditions

[0036] The process line was configured as: -
Premix - Pump - A_1-unit - C_1-unit - A_2-unit
Premix temperature was set at 60°C and 60-rpm stirrer speed. All units were set to 15°C, with shaft speeds set to 1000 rpm. Throughput was 50 g/min. using the constant displacement pump.
[0037] For all products a coarse premix was prepared by slowly adding the prepared aqueous phase to the oil phase in the premix tank. A 2 kg-batch was employed.
The mix was stirred for 15 minutes before pumping. After pumping the line was allowed to run for 15 minutes before any collection of product.
[0038] The following process parameters were noted: -

Product	A_1 exit (°C)	C_1 exit (°C)	A_2 exit (°C)	Line Pressure (bar)
Control	20.2	19.4	17.6	1.0
10% tt CLA	21.4	20.2	18.0	1.4

[0039] All tubs were placed at 5°C. After one day, one tub of each was transferred to each of 5°, 10°, 15° and 20°C for evaluations after one week.
[0040] All samples spread easily with no apparent water loss. All products are of excellent quality and displayed very good values for hardness (C-value), collar and conductivity at all storage temperatures (5, 10, 15 and 20°C).

5°C Storage

[0041]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	630	I/II	$<10^{-5}$
10% tt CLA	610	I/II	$<10^{-5}$

10° Storage

[0042]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	410	I	$<10^{-5}$
10% tt CLA	370	I	$<10^{-5}$

15°C Storage

[0043]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	340	I	$<10^{-5}$
10% tt CLA	390	I	$<10^{-5}$

20°C Storage

[0044]

Sample	C-Value (g/cm^2)	Collar (Scale I to VI)	Conductivity (μScm^{-1})
Control	300	I	$<10^{-5}$
10% tt CLA	280	I	$<10^{-5}$

Example 7 Preparation of ice cream

[0045]

Table 1:

Recipe	
The following recipe was applied (cf table 1)	
Component	Wt%
Fat blend	10.0
Skimmed milk powder	10.0
Crystal sugar	12.0
Clear syrup	4.0
Dextrose anhydrate	2
Dimodan PVP	0.6
Water	61.4

[0046] The fat blends that were used are disclosed in table 2

Table 2:

Fat blend		
	Component	Wt%
Reference	POf IV65/ CN/ SF	30/20/50
Sample	POf IV65/CN/CLA tt containing oil	30/20/50

[0047] The sugar, milk powder and dextrose were mixed and added to the water. The mixture was heated to 70°C and the clear syrup was added. Next the fat blend and the emulsifier were added. The emulsion was stirred with an ultra-turrax, cooled down to 20°C and stirred again with the ultra-turrax. The emulsion stayed overnight in the refrigerator at 7°C. The batch ice cream machine was held for 24 hours at -28°C. The emulsion was stirred in the machine for 40 minutes until the temperature was at its lowest. The resulting ice cream was stored at -18°C for at least 3 days and was then evaluated.

[0048] From table 3 it can be concluded that the products according to the invention displayed a better hardness and a higher overrun.

Table 3:

	Reference	Product with CLA t,t
Overrun in %	6.7	14.4
hardness	52	173

Taste panel

[0049] Seven panellist tasted the firmness of sample in comparison to the reference. For 5 out of seven the sample with CLA t,t had a higher firmness.

1. Reference with SF - CLA tt			
	less/slower	equal	more/faster
Firmness		2	5

Claims

1. Use of a composition comprising 1 to 100, preferably 5 to 90, most preferably 20 to 85 wt% (on total composition) of isomers of trans/trans conjugated linoleic acid (=tt-CLA) as measured by ^{13}C-magnetic resonance, wherein the tt-CLA is applied as anti-inflammatory agent.

2. Use of a composition according to claim 1 wherein the composition, comprising the tt-CLA is applied as an additive in a food product or as food supplement, preferably in encapsulated form.

3. Use of a composition according to claims 1 and 2 wherein the composition, comprising the tt-CLA isomers contains as measured by ^{13}C magnetic resonance techniques 5 to 90 wt% (on total composition) of tt-CLA, while also the CLA isomers cis^{9}trans^{11} and trans^{10}cis^{12} are present in a weight ratio of (total $t^{9}t^{11}+t^{10}t^{12}$) to ($c^{9}t^{11}+t^{10}c^{12}$) of more than 3:1, preferably more than 5:1, most preferably more than 7.5:1.

4. Use of a composition according to claims 1 to 3 wherein the tt-CLA isomers are present either as free fatty acids, or as mono-, di-, and/or triglycerides or as alkylesters.

5. Composition with anti-inflammatory properties comprising CLA isomers, wherein the composition comprises more than 10 wt%, preferably more than 25 wt%, most preferably more than 50 wt% of CLA-isomers, from which more than 55, preferably more than 60, most preferably more than 65 wt% (on total CLA isomers) are tt-CLA isomers as measured by ^{13}C-magnetic resonance techniques.

6. Composition with anti inflammatory properties according to claim 5, wherein the composition comprises the tt-CLA isomers t^9t^{11} and $t^{10}t^{12}$ in a weight ratio t^9t^{11} to $t^{10}t^{12}$ of less than 2.3:1, preferably less than 1.8:1, most preferably less than 1.0:1 (as measures by ^{13}C magnetic resonance techniques).

7. Composition according to claims 5 or 6 wherein the composition also contains c^9t^{11} and $t^{10}c^{12}$-CLA isomers in a weight ratio of (total $t^9t^{11}+t^{10}t^{12}$) to ($c^9t^{11}+t^{10}c^{12}$) of more than 3:1, preferable more than 5:1, most preferable more than 7.5:1 as measured by ^{13}C-magnetic resonance technology.

8. Food products, preferably selected from the group consisting of confectionery products, spreads, sauces, dressings, mayonnaise, cheese, ice cream, doughs, baked bakery products, fillings, creams containing an effective amount of a composition comprising 1 to 100 wt% of tt-CLA, preferably in the form of a mono- and/or di- and/or triglyceride and most preferably containing an effective amount of the composition according to claims 5 to 7.

9. Food supplements containing an effective amount of a composition comprising 1 to 100 wt% of tt-CLA, preferably containing an effective amount of a composition according to claims 5 to 7.

10. Food supplements according to claim 9 wherein the tt-CLA rich composition is encapsulated in a food grade encapsulating material.

11. Food supplement according to claims 9 to 10 wherein the composition comprising the tt-CLA is fortified with other food ingredients selected from the group consisting of vitamins A, B, C, D, E, K, minerals such as calcium, potassium, magnesium, iron, copper, zinc, selenium and anti-oxidants such as tocopherols, polyphenols.

12. Process for the preparation of a composition according to claims 5 to 7, wherein:

    i) a composition with a total unconjugated C18:2 content of more than 55 wt% is conjugated and isomerized by subjecting it to a catalytic treatment with a Sulphur-Nickel catalyst at a temperature of 100 to 180 oC in the absence of hydrogen
    ii) the product formed in step i) is subjected to a solvent fractionation using acetone in a weight ratio of acetone to CLA product of 10:1 to 1:1 at a temperature of -25 to -100 °C, whereupon a stearine (or top) fraction is isolated as product according to claims 5 to 6.

13. Process for the enrichment of a mixture comprising different isomers of conjugated linoleic acid in the tt-CLA isomers wherein a mixture comprising at least 1 wt% of tt-CLA in admixture with other CLA isomers is subjected to an enzymic conversion, selected from the group consisting of i) partial hydrolysis of a glyceride mixture or of a mixture of esters of short chain alcohols, ii) partial esterification of a mixture of free fatty acids with glycerol or with a glyceride, iii) partial glycerolysis of a glyceride mixture and iv) partial esterification of an alcohol with free CLA isomers, while using an enzyme that can discriminate tt-CLA from other CLA isomers, whereupon the reaction product is separated by physical means separating a fraction that is enriched in tt-CLA as reaction product.

14. Process according to claim 13 wherein the enzyme is selected from the group consisting of: Candida rugosa lipase, Lipase QL, Lipase SL, Lipase OF, Geotrichum candidum B lipase, Lypozyme IM and lypozyme M.

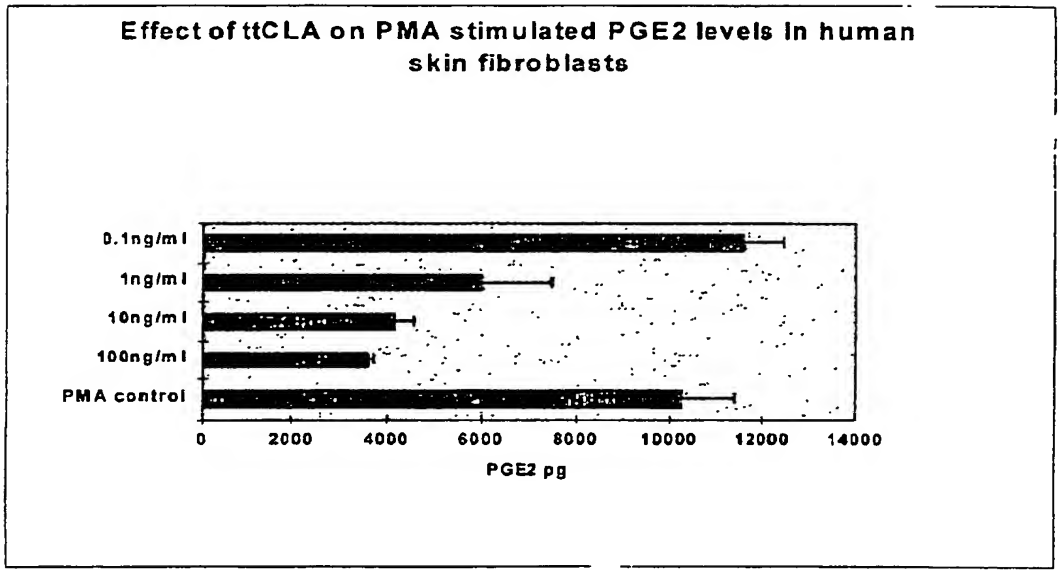

Fig. 1 Effect of ttCLA on PGE2 levels.

European Patent Office

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Application Number
EP 00 20 3510

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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X : particularly relevant if taken alone
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D : document cited in the application
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EUROPEAN SEARCH REPORT

Application Number
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